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Abstract

Connexin proteins oligomerize in hexameric structures called connexin hemichannels, which then dock to form gap junctions. Gap junctions direct cell-cell communication by allowing the exchange of small molecules and ions between neighboring cells. In this way, hepatic gap junctions support liver homeostasis. Besides serving as building block for gap junctions, connexin hemichannels provide a pathway between the intracellular and the extracellular environment. The activation of connexin hemichannels is associated with acute and chronic liver pathologies. The present review discusses the role of gap junctions and connexin hemichannels in the liver.

Didactic synopsis

- Gap junctions, consisting of connexin proteins, foresee a pathway for intercellular communication between liver cells allowing the passage of small molecules and ions between adjacent cells.
- Gap junctions are considered as key players in liver homeostasis. They support several liver-specific functions, such as the biotransformation of xenobiotics, bile production, the synthesis and secretion of proteins and the control of carbohydrate metabolism.
- Activation of connexin hemichannels is associated with acute and chronic liver diseases.

Didactic legends

See attachment.

Introduction

Connexin (Cx) proteins are expressed by many types of cells across multiple tissues of the human body (45, 174). More than 20 different Cx species have already been identified (45). Cx proteins are named based on their molecular weight. In this regard, the most abundantly expressed Cx variant is referred to as Cx43, as cDNA sequencing predicts a molecular weight of 43 kDa for this Cx protein (73). Nevertheless, all Cx species have structural similarities. They are defined as multi-pass transmembrane proteins spanning the cell plasma membrane 4 times (Figure 1). In addition to 4 transmembrane regions, each Cx protein consists of 2 extracellular loops, 1 cytoplasmic loop, an intracellular carboxy and an amino terminus (73, 145). Cx proteins contribute to cellular communication by acting as structural precursors of Cx-based channels, *i.e.* Cx hemichannels and gap junctions. Cx proteins are synthesized by membrane-bound ribosomes, and are co-translationally integrated into the endoplasmic reticulum (73, 145). Subsequently, 6 Cx proteins oligomerize forming Cx hemichannels. The localization of oligomerization is Cx-dependent. Cx26 and Cx32 gather together in the endoplasmic reticulum, while Cx43 forms Cx hemichannels in the Golgi apparatus. Such oligomerization of Cx proteins creates 2 types of Cx hemichannels. Homomeric hemichannels consist of 6 Cx proteins of a single type, whereas heteromeric hemichannels are composed of different types of Cx proteins (9, 73). Gap junctions arise from the interaction of 2 Cx hemichannels on adjacent cells. Gap junctions are classified according to the composition of Cx hemichannels. Identical or homomeric Cx hemichannels form homotypic gap junctions, whereas 2 heteromeric and 2 different Cx hemichannels form heteromeric and heterotypic gap junctions, respectively (9, 73). These cell-cell junctions mediate passive intercellular diffusion of small and hydrophilic molecules (1-1.5 kDa), including glucose, glutamate, glutathione, adenosine triphosphate, cyclic adenosine monophosphate, inositol triphosphate and ions, such as calcium, sodium and potassium (9, 73, 103, 145). The cross-membrane trafficking of these

substances is essential to control numerous processes. In this regard, the well-orchestrated exchange of biomolecules and ions between hepatic cells dictates crucial cellular actions for optimal liver functioning (103, 159, 177). Consequently, Cx proteins and their channels are indispensable in the liver (103). In the present paper, the role of Cx channels in normal hepatic functionality, hepatic life cycle and liver diseases is described.

Liver lobule organization

The liver is the largest solid organ in the human body. The liver serves as the gatekeeper between the digestive tract and the rest of the body (2, 4, 66, 177). The rib cage forms a protective shield around the body's vital organs, including the liver. The liver is located in the upper right side of the abdomen below the diaphragm. Because blood from the small intestine, spleen, stomach, and pancreas can contain drugs, toxins or pathogens, foreign material first enters the liver before being released into the circulatory system. The liver's primary function is to aid in detoxification by metabolizing and breaking down xenobiotics (4). The liver can do this due to the supply and removal of blood *via* the hepatic artery, hepatic portal vein and hepatic vein, and the production of bile (Figure 2) (31). Branches of the hepatic artery, hepatic portal vein and bile duct form the portal triad (99). These portal triads are in close contact with hepatocytes, being the parenchymal cells of the liver. Hepatocytes make up the bulk of the liver mass and are organized in hexagonal structures called hepatic lobules. Hepatocytes build hepatic cellular plates that diverge from the central hepatic vein. The interaction between portal triads and hepatocytes occurs at the sinusoidal or basal membrane pole of the cells (177, 178). Branches of the hepatic artery and portal vein join in hepatic sinusoids that supply blood to hepatocytes. These sinusoids have no basal membrane and are enclosed by fenestrated sinusoidal endothelial cells, providing a closely interaction between blood and hepatocytes (Figure 3). After a bidirectional exchange of substances, blood is drained to the central hepatic

vein (177, 178). By doing this, a mixture of oxygen and nutrient-rich blood reaches the hepatocytes. In this regard, the localization of hepatocytes in the hepatic lobule determines access to oxygen and nutrients. The canalicular network divides the acinus, the functional unit of the liver, into metabolic zones, namely the periportal, midlobular, and perivenous zone (31). Hepatocytes secrete bile into the bile canaliculi at their canalicular site (19). Next to blood and bile flow, there is drainage of lymph fluid in the liver. The large gaps between liver sinusoidal cells cause a massive release of protein-rich fluid into Disse's space, also known as the perisinusoidal space. The space of Disse is located in between sinusoids and hepatic cellular plates, and allows free contact between released tissue fluid and the sinusoidal site of hepatocytes (150). Following interaction between the hepatocytes and the extracellular environment, hepatic tissue fluid exits the hepatic lobule. Hepatic tissue fluid can be reabsorbed in the sinusoids or leave the liver as lymph fluid *via* lymphatic vessels (150, 177). In addition to hepatocytes and liver sinusoidal endothelial cells, the liver also harbours macrophages and stellate cells. The liver-resident macrophages, known as Kupffer cells, reside in the sinusoidal lumen. They are attached to the liver sinusoidal endothelial cells, and play an essential role in the host defense mechanism by trapping and phagocytosing foreign substances. Hepatic stellate cells, also called Ito cells, are located in the perisinusoidal space (177). Under physiological conditions, these cells show vitamin A-rich droplets. Hepatic stellate cells also produce extracellular matrix components, matrix metalloproteinases and regulate the sinusoidal blood flow (48, 84).

Hepatic connexin proteins

Liver homeostasis is controlled by extracellular, intracellular and intercellular communication mechanisms (27). Cx proteins are acknowledged as key players in the regulation of tissue homeostasis. Gap junctions foresee intercellular communication by allowing direct cell-to-cell

communication (27, 179, 181). In addition to acting as structural precursors of gap junctions, Cx hemichannels mediate communication on their own as well. Cx hemichannels drive cellular communication between the intracellular compartment and extracellular environment. Open Cx hemichannels allow the passage of substances that show great overlap with those involved in gap junction intercellular communication (27, 179, 181). Of note, Cx proteins also participate in managing tissue homeostasis independently of their channel properties. Such non-channel actions of Cx proteins impact cell growth and cell death. Thus, Cx proteins can interact directly with cell growth and cell death regulators. In this regard, interactions of Cx proteins with β-catenin, E-cadherin, zonula occludens 1-associated nucleic acid binding protein, zonula occludens proteins, nephroblastoma overexpressed protein, discs-large homolog 1 protein, B-cell lymphoma-2 proteins and apoptosis signal-regulating kinase 1 modulate cell growth and cell death mechanisms. These protein-protein interactions typically occur at the cytoplasmic carboxy tail of Cx proteins (179, 181). These non-canonical roles of Cx proteins also may involve direct effects on the transcription of genes involved in the regulation of cell growth or cell death (179, 181).

Various Cx species are detected in the liver (Figure 4), but Cx32 is the predominant one, representing 90% of the total hepatic Cx protein content. Cx32 is expressed by hepatocytes and to a lesser extent by sinusoidal endothelial cells and cholangiocytes, which are epithelial cells that line bile ducts (103, 184). Cx43 protein is also widespread as it is detected in cholangiocytes, Kupffer cells, hepatic stellate cells, hepatic artery, portal vein and sinusoidal endothelial cells. Furthermore, Cx37 and Cx40 proteins are present in the liver vascular cells. They can be found in endothelial cells of the hepatic artery and portal vein (55, 103, 184). Cx26 is another Cx family member expressed in the liver. Cx26 is produced by hepatocytes, hepatic stellate cells and sinusoidal epithelial cells. The occurrence of Cx26 protein appears to be subject to by liver zonation effects, as this protein is preferentially expressed by hepatocytes in

the periportal region (103, 184). Cx proteins are also expressed at the mitochondrial membrane. Cx32 proteins are present at the inner mitochondrial membrane of hepatocytes, where they can directly interact with other mitochondrial proteins (47). Mitochondrial Cx43 proteins are detected in mouse liver tissue (94). Nevertheless, the role of Cx proteins in this subcellular organelle is still unclear.

Cx proteins are short-lived. Hepatic Cxs have a half-live of just a few hours. The surprisingly high turnover rate of Cx proteins is associated with proteasomal and lysosomal degradation mechanisms. Proteasomal activity stimulates the internalization of Cx proteins from the cell plasma membrane. Cx proteins arise in the cytoplasm by the formation of vesicle-like double membrane structures. The fusion of endocytosed membrane vesicles with lysosomes further degrades Cx proteins (43, 51).

Despite different types of liver cells expressing Cx proteins, functional gap junctions are only seen in hepatocytes and hepatic stellate cells. Gap junctions cover approximately 3 % of the cell plasma membrane surface of hepatocytes. These gap junction channels, which are typically 15 Å in diameter and 180 Å in length, are organized into plaques containing 10 to 10,000 channels and scale 0.2 to 1 μm in diameter (103, 184).

Regulation of gap junctions in liver

Gap junction regulation is controlled at both functionality and expression levels. Control at the functionality level typically relies on short-term regulatory mechanisms. These gating mechanisms mainly include posttranslational modifications (1, 119). The most abundantly described posttranslational modification of Cx proteins is phosphorylation (1). Cx phosphorylation usually occurs at serine, threonine and tyrosine residues of the carboxy terminus (92). Multiple enzymes are involved in Cx phosphorylation in hepatocytes, such as protein kinase B, protein kinase C, cyclic adenosine monophosphate-dependent protein kinase

and calcium ion/calmodulin-dependent protein kinase II (129, 144). Additional posttranslational modifications include methylation, SUMOylation, ubiquitination, glycosylation, tyrosination and acetylation (92, 155). Other short-term regulatory mechanisms encompass changes in intracellular calcium ion levels, pH, redox potential and transmembrane voltage (11, 119, 127, 128, 130). Calcium ion-mediated gating relates to the interaction of Cx proteins with calmodulin. Calmodulin hereby provides a physical barrier hindering gap junction opening by binding to the cytoplasmic loop or amino terminus of Cx proteins. This conformational change occurs upon binding of calcium ions, and restores gap junction activity (128). The pH-mediated gating mechanism also involves conformational changes whereby gap junctions remain closed in acidic conditions, but open at alkaline pH (49, 71).

Control at expression level is regulated by the transcriptional machinery, including *cis/trans* and epigenetic mechanisms (119). Hepatocyte nuclear factor 1 alpha and the ubiquitously expressed specificity protein 1 both are transcription factors linked with Cx32 protein expression in the liver (75). Epigenetic mechanisms include DNA methylation and histone acetylation (119). DNA methyltransferases inhibit gene transcription by hypermethylation of gene promotors. Hypermethylation of CpG dinucleotides in its gene promotor lowers Cx26 expression in human hepatocellular carcinoma (HCC) tissue (157, 167). Histone acetyltransferases and deacetylases are other known epigenetic modifiers (119). These enzymes control gene transcription *via* decondensation and condensation of chromatin, respectively. Inhibition of histone deacetylases increases Cx32 protein expression in primary rat hepatocytes resulting in increased gap junction intercellular communication (185, 186). Similarly, enhanced gap junction activity is observed in rat liver epithelial cells upon inhibition of histone deacetylase activity (65). In Huh7 cells, Cx43 protein levels are lower due to the inhibition of histone deacetylase activity (197).

Role of gap junctions in liver-specific functionality

Biotransformation of xenobiotics

Xenobiotics undergo biotransformation in the liver, protecting the body from harmful exogenous compounds. During this process, xenobiotics are transformed to reduce their toxicity and become more hydrophilic. Foreign substances, such as drugs, toxins, biocides and food additives undergo phase I and phase II reactions (40). Phase I involves oxidation, reduction and hydrolysis reactions that aim to break down exogenous substances into less toxic metabolites. Phase II reactions further convert such intermediate metabolites to increase their water solubility and facilitate renal excretion. Phase II consists of mechanisms, like glucuronidation, sulfation, acetylation, methylation, and conjugation with glutathione and amino acids, forming conjugated metabolites (40, 149). Biotransformation is catalyzed by the liver enzymes mainly found in hepatocytes, including cytochrome P450 enzymes, flavine-containing monooxygenases, esterases, amidases, epoxide hydrolases and uridine diphosphate glucuronosyltransferase (40, 149). Cx proteins influence biotransformation reactions in the liver (Figure 5). *In vitro* studies with WB-F344 cell line derived from an adult rat liver with hepatic progenitor-like properties show a correlation between Cx protein expression and cytochrome P450 activity. Treatment of WB-F344 cells with a p38 mitogen-activated protein kinase inhibitor promotes hepatocyte differentiation. This goes pairwise with the overexpression of Cx32 and knockdown of Cx43 proteins (126). When WB-F344 cells differentiate into hepatocytes, induced activity of cytochrome P450 3A4 is noted (126). Similarly, an association between Cx proteins and cytochrome P450-mediated biotransformation is seen in primary rat hepatocytes. Co-cultivation of isolated rat hepatocytes with mouse embryonic fibroblast cells increases cytochrome P450 activity. This co-cultivation of rat hepatocytes also induces gap junction activity through the appearance of Cx26 and Cx32 between cultured hepatocytes and fibroblasts (72). Furthermore, inhibition of gap junction

intercellular communication suppresses cytochrome P450 activity in hepatocyte/fibroblast co-cultures (72).

Production of bile

Bile production takes place in hepatocytes and cholangiocytes. Bile is critical for digestion of food by mediating absorption of fats in the duodenum (19, 208). Although bile can be reabsorbed and returned to the liver through the portal vein, the human body must replenish its daily loss. As such, bile salts are formed upon oxidation of cholesterol through enzymatic reactions. Cytochrome P450 7A1 plays a crucial role in this regard because it is involved in the catabolism of cholesterol by converting cholesterol to 7-α-hydroxycholesterol, being the rate-limiting step in bile acid formation (22, 23). Next to cytochrome P450 activity, the regulation of the uptake and excretion of bile salts by hepatocytes is essential for bile production. Transporters on the basolateral surface of hepatocytes control ion flux to allow uptake of bile salts. The activity of transporters at the canalicular site, which is mainly dependent on adenosine triphosphate, mediates the outflow of bile components into bile collection channels (23, 33). Cx proteins are involved in bile production (Figure 5) since the process of bile secretion is controlled by calcium ions crossing between hepatocytes *via* intercellular communication pathways (175). Gap junctions control the contraction of bile canaliculi from midlobular to periportal hepatocytes. In fact, reduced bile flow to the hepatic bile duct is noted for Cx32-deficient mice compared to wild-type counterparts (169). In addition, a decrease in Cx26 protein expression is observed in liver extracts derived from Cx32-deficient mice (169). Hepatocytes provide a physical barrier between hepatic sinusoids and bile canaliculi to prevent the mixing of blood and bile. This barrier mainly consists of tight junctions on the canalicular side of hepatocytes, but other cell-cell junctions, including adherens junctions, desmosomes, and gap junctions, are involved as well (81, 132). In this light, zonula occluding proteins

colocalize with Cx32 proteins in rat hepatocytes (76). Similarly, a correlation between tight junctions and Cx32 proteins is seen in mouse hepatocytes and human Cx32-transfected mouse hepatocytes show induced expression of tight junction proteins (77). In addition, forcing the expression of Cx32 proteins increases tight junction formation and function. The role of functional gap junctions is important because their inhibition leads to a reduction of tight junction proteins presence on the cell plasma membrane surface (78).

Synthesis and secretion of proteins

Human proteins are made up of 20 kinds of amino acids. Half of these amino acids can be synthesized in the liver. These so-called non-essential amino acids and includes alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine and tyrosine. The remaining 10 amino acids, referred to as essential amino acids, must be supplied through the diet (36, 56, 90). All individual amino acids are necessary for the successful synthesis of hepatic protein products, including acute phase proteins, clotting factors, fibrinolytic proteins and protease inhibitors (124). Cx proteins are involved in the production and secretion of proteins in the liver (Figure 5). Transfection of human hepatoma HepG2 cells with plasmid encoding human Cx32 results in enhanced albumin (199).

Carbohydrate metabolism

Many of the carbohydrate metabolism reactions take place in the liver. Thus, the liver plays a vital role in maintaining energy balance. The processes of carbohydrate metabolism are regulated by the feed-fast cycle and energy requirements. The digestion of carbohydrates through glycolysis contributes to the supply of acetyl coenzyme A. As these acetyl coenzyme A molecules are further converted by the Krebs cycle to generate adenosine triphosphate, the breakdown of carbohydrates is responsible for the formation of energy-rich molecules (53, 63).

The liver performs carbohydrate metabolism. It involves glycogenolysis and glycogenesis, the breakdown and synthesis of glycogen, respectively. While glycogenolysis relates to the catabolism of glycogen into glucose molecules that can be further converted by glycolysis, glycogenesis allows storing and conserving energy for future use by linking glucose molecules. Moreover, the liver also directs the formation of glucose or gluconeogenesis (13, 53, 63, 131). Liver perfusion experiments with Cx32-deficient and wild-type mice show that intercellular communication mechanisms between hepatocytes regulate the propagation of hormonal signals to stimulate the release of glucose from the liver (Figure 5). Cx32-deficient animals exhibit aberrant release of glucose. Cx32 proteins build up gap junctions that enhance the diffusion of noradrenaline and glucagon-induced signals from periportal to perivenous hepatocytes to organize glucose release under physiological conditions (162). These channels are essential to compensate for the degradation effects on blood circulating hormones. Hormones undergo degradation on their way from the periportal towards the perivenous zone. As a consequence, hormone action is less pronounced in the perivenous region, and the hepatocyte-mediated glucose release gradually decreases along the sinusoids. Hepatic gap junctions may partially compensate for this by enhancing hepatic glucose release through the diffusion of noradrenaline and glucagon-induced signals between hepatocytes (162).

Role of gap junctions in the hepatic life cycle

Liver cell proliferation

Hepatocytes display a very low turnover rate (187). However, the liver displays a strong regenerative capacity upon partial hepatectomy (136). The latter is compromised during chronic liver diseases, but the liver can still regenerate due to the presence of adult liver progenitor cells (136). *In vivo* regeneration of rat liver upon partial hepatectomy shows that Cx26, Cx32 and Cx43 display a unique spatiotemporal expression pattern during liver cell

proliferation (118). Whereas Cx26 protein expression is normally restricted to periportal hepatocytes, induced Cx26 protein expression pattern is observed in hepatocytes located in the midlobular and perivenous zone (118). This elevated production of Cx26 proteins is seen until the onset of the S-phase and is associated with colocalization of Cx26 and Cx32 proteins (118). Furthermore, immunostaining also shows a redistribution of Cx32 proteins upon partial hepatectomy as the typical punctuated signal pattern at the cell plasma membrane surface changes into a more diffuse fluorescence signal (118). Gap junction activity plays an elusive role in cell growth (Figure 6). It typically increases in the G1 phase and then suddenly decreases at the beginning of the S-phase. This pattern is demonstrated in both *in vitro* and *in vivo* studies (37, 46, 80, 82, 85, 107, 108, 163, 168, 176). The progression from the G1 to the S phase seems to be linked with decreased levels of Cx protein expression and concomitantly reduced gap junction activity (85, 168, 176). *In vitro* data show that induction of cell proliferation in liver cells is associated with decreased Cx26 and Cx32 protein levels, probably mediated *via* increased proteasomal degradation or reduced mRNA stability (85, 168, 176). Similarly, an *in vitro* model of hepatocyte proliferation, namely mitogen-stimulated primary hepatocytes, revealed that decreased Cx32 expression is linked with mitogen-activated protein kinase activity (46, 79, 80). In rat liver cells, protein kinase C-dependent phosphorylation of Cx43 disrupts gap junction communication mechanisms upon progression from the G0 to the S phase (83). Although Cx levels are generally downregulated during the onset of the S phase, restoration of gap junction activity does not affect growth in cultured human HCC cells (39). This indicates that although gap junction intercellular communication is downregulated and related to proliferation, it is not the cause for hepatocyte proliferation (46). More likely, it is responsible for allowing cell cycle progression instead of being the initiator of proliferation (68).

Liver cell differentiation

Gap junctions play a crucial role in liver cell differentiation (Figure 6). Oval cells, which are liver stem cells that can differentiate into hepatocytes, show a strong expression of Cx43 proteins and a decreased Cx32 protein expression *in vivo* (205). However, a clear switch from Cx43 to Cx32 is seen during differentiation, both *in vitro* and *in vivo* (60). Vitamin K2 stimulates hepatocyte differentiation and maturation of human embryonic stem cells *via* enhanced Cx32 expression and gap junction activity, whereas β-carotene and vitamin A delay proliferation and Cx43 expression during hepatocyte differentiation in the rat (115, 134). In embryonic livers, Cx patterns are lineage-stage dependent (126). Timelines vary among studies, but Cx26 and Cx32 proteins become detectable during late gestation in rats, and both their expression levels reach their maximum at 1 week to 3 weeks after birth (60). When these expression levels are reached, gap junction activity is elevated and Cx proteins display a distinct distribution pattern (18, 60). While Cx32 is uniformly distributed among hepatocytes, the presence of Cx26 proteins is more pronounced in the periportal area (18, 60, 116). This zonation pattern increases and upon complete polarization of the hepatocytes, Cx26 is hereby primarily present at the periportal area due to presence of glucagon, which induces Cx26 mRNA expression (18, 60). In accordance with the differentiation expression patterns of Cx proteins seen in stem cell-based regenerative processes and during gestation, Cx32 is frequently used as a functional hepatic marker in stem cell-based hepatocyte differentiation studies (38, 70, 97, 140, 160, 205). The aim of these studies is to form functional hepatocytes from stem cells, such as umbilical cord matrix stem cells, peritoneal adipose mesenchymal stem cells, human embryonic stem cells or oval cells, and to use differentiated cells as *in vitro* models or for therapeutic purposes (21, 134, 153, 173). Not only stem cells, but also epithelial cells are employed to recreate hepatocytes *in vitro* or *in vivo* (89, 91). While Cx32 acts as a late hepatic differentiation marker, Cx43 is used as an early hepatic marker to characterize

transdifferentiation processes or to classify hepatic cell lines according to their differentiation stage (34, 141). Similarly, Cx32 is used as a marker in organoid experiments and in studies that optimize primary hepatocyte cell cultures by postponing cell dedifferentiation (15, 59). In fact, the opposite switch from Cx32 and Cx26 to Cx43 in Cx expression patterns is noted during hepatocyte dedifferentiation in liver disease and primary hepatocyte cultures (195).

Liver cell death

The involvement of Cx signaling in liver cell death has been investigated in various *in vitro* systems (26, 67, 88). Most studies point to an apoptosis-mediating role for Cx channels (Figure 6). In the liver, Cx channels have been associated with different types of cell death, such as apoptosis, necrosis and autophagy (14, 95, 105, 147, 165, 182). Gap junction intercellular communication can be modulated *via* autophagy-based degradation of Cx proteins in response to physiological stimuli. At the same time, gap junction activity itself is involved in autophagy induced dell death (95). During the early stages of apoptosis in serum-deprived WB-F344 cells, gap junction activity increases because it is responsible for the propagation of the calcium ion-mediated death wave (193). Following these initial stages, intercellular communication *via* gap junctions declines (193). Similarly, Cx43 channel activity plays a role in the initiation of spontaneous apoptosis in primary hepatocyte cultures (183). Early elevated gap junction activity is attributed to increased expression and phosphorylation of Cx43 (193). The latter is probably mediated by the cyclin-dependent kinase 1/cyclin B complex, while increased protein expression is associated with elevated mRNA expression of Cx43 caused by histone H3/H4 acetylation (121, 193). Additionally, overexpression of Cx26 proteins induces gap junction activity in human HCC cells and reduces their malignant potential by inducing apoptosis (110). In general, the apoptosis-mediating function of gap junctions is linked with their ability to spread cell death factors, in particular inositol triphosphate (61). Some studies described a

decrease in gap junction activity during apoptosis and thus assign a protective role to Cx proteins and their corresponding channels (10, 57, 58, 62, 104, 105). Lowering gap junction activity in these conditions is not solely attributed to reduced Cx expression. In rat liver epithelial cells, inhibition of gap junction activity is accompanied by hyperphosphorylation of Cx43 mediated by mitogen-activated protein kinases and aberrant localization of Cx32 or Cx43 (5, 17, 133).

Role of connexin hemichannels in liver disease

In recent years, it has become clear that Cx hemichannels can foresee a pathway for cellular communication on their own, independent of their role as building stones of gap junctions, albeit between the intracellular compartment and the extracellular environment. Functional Cx hemichannels are present in hepatocytes (55, 103, 184). Since activation of Cx hemichannels drives inflammation and cell death, Cx hemichannels are associated with to the onset and spread of liver disease (180). In this regard, opening of hepatic Cx hemichannels has been reported to underlie acute liver injury, infectious hepatitis, cholestasis, non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and HCC (Figure 7).

Acute liver injury

Drug-induced liver injury is the most common cause of acute liver failure. An overdose of acetaminophen (APAP) is known to cause loss of hepatocyte function (74, 161). APAP is regularly used as an analgesic or antipyretic drug, but APAP-metabolite adducts can cause liver injury. In overdose, *N*-acetyl-*p*-benzoquinone imine, a metabolite of APAP, accumulates and causes mitochondrial dysfunction. This is accompanied by an excessive amount of hepatocellular cell death, *i.e.* necrosis, which triggers an inflammatory response in the liver (135, 198). Cx proteins are implicated in acute liver injury. In this regard, *in vivo* experiments

demonstrate that APAP-induced liver injury causes drastic changes in hepatic Cx expression profiles (104). After injection of APAP, Cx26 and Cx32 protein levels decrease significantly in murine liver. On the other hand, hepatic Cx43 protein levels increase (104). Not surprisingly, these changes in Cx expression are accompanied by a change in gap junction activity. Dye transfer experiments indicate a decrease of hepatic gap junction activity following APAP overdosing (104). Similarly, acute carbon tetrachloride (CCl_4)-induced liver damage causes changes in Cx expression in rats. Accordingly, hepatic Cx26 and Cx32 protein levels decrease, while Cx43 protein expression increases during CCl_4-induced liver damage (143). Such changes may be associated with a deterioration in the activity of gap junctions, which contributes to hepatic dysfunction. Cx32 and associated gap junctions are found to play an important role during APAP-induced hepatotoxicity in rats (111). It has been reported that Cx32-based gap junctions activity is involved in the progression of liver injury in thioacetamide-treated mice (125). While gap junction activity controls the removal of damaged hepatocytes, the induced expression of Cx43 proteins contributes to APAP-induced hepatic cell death processes by propagating cell death signals (111). Moreover, experiments with TAT-Gap19 and TAT-Gap24 show that Cx hemichannels play a prominent role during acute liver injury (102). TAT-Gap19 and TAT-Gap24 are peptide-based inhibitors of Cx43 and Cx32 hemichannels, respectively. Both peptides reproduce specific amino acid sequences in the structure of Cx proteins to selectively block Cx hemichannel opening. *In vivo* experiments with these peptides demonstrate that blocking Cx-based signaling reduces APAP-induced liver injury in mice. Treatment with TAT-Gap19 and/or TAT-Gap24 causes a decrease in necrotic areas in the liver and reduces APAP-induced inflammation (102).

Infectious hepatitis

Infectious hepatitis includes inflammation of the liver parenchyma (96). Based on the duration of the inflammation, hepatitis can be classified as acute or chronic hepatitis (96). While hepatitis caused by hepatotrophic viruses results primarily in acute inflammation, several of them, particularly hepatitis B, C, D, and E, can burgeon into chronic hepatitis (137, 158). The latter can even lead to the development of HCC (98, 166, 189). Cx32 protein expression is lowered in patients with chronic hepatitis and gradually decreases as the pathology progresses towards liver cirrhosis and HCC (113, 196). In addition, Cx32 is internalized and becomes irregularly distributed in the parenchyma (113, 196). During acute inflammation induced by lipopolysaccharides (LPS) in rats, hepatic Cx32 mRNA and protein levels are lower and gap junction activity is impaired (29, 35, 50, 171). Similarly, a decline of Cx26 and Cx32 levels is observed in primary human hepatocytes treated with LPS (201). This results in an attenuation of gap junction intercellular communication. Cx levels can be stabilized and gap junction activity can be restored by exposure to gadolinium chloride, which has been reported to protect against liver inflammation by inactivating Kupffer cells (201). Cytokines also affect Cx32 protein levels. Treatment with interleukin-1, interleukin-6, or tumor necrosis factor-alpha downregulates Cx32 expression in immortalized mouse hepatocytes. Such conditions of experimental inflammation affect gap junction-mediated communication (170). While liver inflammation leads to a decrease of Cx32 proteins, Cx43 expression levels are enhanced. Indeed, an increase in Cx43 protein is seen in rats treated with LPS. This induces the presence of Cx43 proteins in the liver, which is mirrored by the formation of Cx43 gap junctions between rat Kupffer cells. The latter is also observed in cultures of primary rat Kupffer cells upon LPS and interferon gamma-treatment (41).

Cholestasis

Cholestasis denotes any situation whereby bile flow is impeded with a concomitant accumulation of bile acid in hepatocytes or systemic circulation (6, 142). Based on the localization of obstruction, cholestasis can be divided into 2 types, namely intrahepatic and extrahepatic cholestasis. One of the most well-known causes of intrahepatic cholestasis is drug treatment. Extrahepatic cholestasis is caused by obstructions in the bile ducts, including tumors and gall stones (122, 123, 156, 188, 200). Expression of Cx26, Cx32 and Cx43 proteins is modified in different models of cholestasis. During obstructive cholestasis (extrahepatic cholestasis), LPS-evoked hepatocellular cholestasis (intrahepatic cholestasis) and choledochocaval fistula (extrahepatic cholestasis) in rats, a decrease in Cx26 and Cx32 proteins is observed in the liver (52). In general, cholestasis causes a downregulation of Cx26 and Cx32 production and an increase of Cx43 expression (28, 44, 52). Dye coupling experiments with cultured rat hepatocytes show that hepatocyte gap junction activity is impaired during various forms of cholestasis (44, 52). The absence of Cx32 is also associated with a different response to LPS-induced cholestasis. In this regard, cholestasis is more pronounced in Cx32-deficient mice, as higher alkaline phosphatase levels are measured in serum compared to wild-type animals (29). Changes in Cx expression and gap junction functionality during cholestasis are often associated with inflammatory responses (10, 44, 52).

Non-alcoholic steatohepatitis

NASH is a progressive form of non-alcoholic fatty liver disease. An excessive accumulation of fatty acids in hepatocytes can cause NASH. This development towards NASH may be initiated by several mechanisms, including insulin resistance, hormones secreted by the adipose tissue, dietary factors, gut microbiota, genetic and epigenetic factors (20, 64). These triggers evoke liver inflammation, which fuels the transition from non-alcoholic fatty liver disease to NASH (152). Cx32 proteins are involved in the progression of NASH (100, 172). Given that

mice deficient in Cx32 show more pronounced liver damage, inflammation, and oxidative stress upon receiving a choline-deficient high fat diet, Cx32 has a protective role in NASH (100, 172). In addition, NASH affects the expression levels of hepatic Cx proteins. Rats fed a methionine-choline-deficient diet show reduced expression of Cx26 and Cx32 (146). Likewise, a cohort study shows that patients with NASH show lower expression of Cx32 in the liver than healthy controls (100). Reduced function of hepatic gap junctions is observed in transgenic rats carrying a dominant negative mutant of Cx32 (8). These transgenic rats are also more susceptible to NASH as an enhancement of NASH-related hepatotoxicity is noted when feeding a methionine-choline-deficient diet (112). Mice fed a choline-deficient high-fat diet benefit from treatment with peptide-based inhibitors of Cx hemichannels. After treatment with TAT-Gap19 and TAT-Gap24, these animals show decreased levels of liver lipids and inflammation, suggesting that Cx hemichannels act as pathological pores in NASH (192).

Liver fibrosis

Chronic liver damage, such as in hepatitis, cholestasis, or NASH, can eventually lead to liver fibrosis. Abnormal wound healing initiates liver fibrosis (3, 12). A correlation exists between Cx proteins and transformation to liver fibrosis. Cx32 dysfunction drives NASH progression to fibrosis through activation of the nuclear factor-κβ pathway and c-Jun amino-terminal kinases (112). Furthermore, a critical role for hepatic Cx32 proteins is demonstrated by injecting CCl_4 into rats. Chronic application of CCl_4 is often used to model liver fibrosis. The CCl_4-induced liver fibrosis in rodents shows a decrease in Cx32 protein expression (114, 151). Similarly, altered expression of hepatic Cx32 proteins is observed in rats after oral administration of dimethylnitrosamine. The latter elicits a fibrotic response accompanied by a change in the location of Cx32 proteins. Immunohistochemical analysis demonstrates that Cx32 proteins form gap junctions on the cell plasma membrane surface of hepatocytes in

control animals. This expression pattern is disrupted in liver fibrosis as a shift in localization of Cx32 proteins towards the cytoplasm is seen in animals treated with dimethylnitrosamine (139). Cx32 deficiency increases liver fibrosis and hepatocellular damage after CCl_4-induced liver injury. In addition, a more severe oxidative stress response is seen in Cx32-deficient mice, underscoring the more prominent manifestation of liver fibrosis in Cx32 knock-out mice (25). Cx43-driven signaling also regulates liver fibrosis. The use of a CCl_4-based fibrosis model in wild-type and heterozygous Cx43 mice shows that a decrease of Cx43 proteins affects the regulation of hepatic fibrogenesis. Deficiency in Cx43 enhances hepatic fibrosis after CCl_4 injury (24). This is also evidenced by treating mice with TAT-Gap19 and carbenoxolone, the latter being an inhibitor of both Cx hemichannel and gap junctions. Treatment with TAT-Gap19 and carbenoxolone reduces liver damage (30). Liver fibrosis is a precursor to more severe states of liver injury, including cirrhosis and HCC (3, 207). Gap junctions, primarily composed of Cx40 and Cx43, appear to be affected by cirrhosis. The activity of gap junctions is lower in cirrhotic rat livers compared to controls. This is reflected by an alteration of hepatic Cx expression, as Cx40 and Cx43 protein quantities are reduced in cirrhotic liver (54). The attenuation of gap junction activity during liver cirrhosis is linked to the manifestation of portal hypertension, a major complication of cirrhosis that may necessitate liver transplantation (16, 54, 55).

Hepatocellular carcinoma

HCC is the most common form of primary liver cancer, representing more than 75% of all cases (164). It often develops in the background of chronic liver diseases such as liver fibrosis or cirrhosis (7). Cx channels play a role in these pathologies and consequently in HCC (113). Gap junction intercellular communication is generally reduced in HCC, as seen in human liver cancer cell lines, human HCC tissue and rat HCC tissue (86, 87, 93, 106). Gap junction activity

517 is considered to be anti-tumorigenic and crucial for normal hepatocyte growth patterns (32, 39,
518 101, 109). In this respect, induced overexpression of Cx32 counteracts HCC proliferation in
519 SMMC-7721 cells (39). This protective role for Cx32 is also seen *in vivo*, as downregulation
520 of Cx32 is linked with a poor prognosis for HCC patients (206). Upregulation of Cx43 gene
521 and/or protein expression in HCC has been documented in studies using human HCC liver
522 biopsies, liver cancer cell lines from human and rat origin as well as *in vivo* models of HCC
523 (86, 93, 106, 117, 120, 191). The aberrant localization of Cx43 and the Cx43 protein expression
524 levels are both inversely correlated with gap junction activity and hence positively correlated
525 with the HCC malignancy (69, 204). In accordance with this tumor-promoting role in HCC
526 patients, suppression of Cx43 in rat HCC cells lines reduces their invasiveness and migration
527 *in vitro* as well as their *in vivo* metastatic potential (120). Although various studies point
528 towards a pro-tumorigenic role for Cx43, other studies have contradicted these findings. In this
529 respect, Cx43 is found to be downregulated in HCC cell lines or human HCC tissue, and is
530 believed to slow down metastasis formation, lower vascular tumor thrombosis and delay early
531 relapse of patients with hepatitis B virus-related HCC (101, 189). Moreover, Cx43 expression
532 is linked with overall survival after radical hepatectomy in patients with hepatitis B virus-
533 related HCC (189). These anti-tumorigenic effects of Cx43 could be mediated through its
534 regulating effect on the expression of cancer-related genes belonging to Fc gamma receptor-
535 mediated phagocytosis, neurotrophin signaling, angiogenesis and calcium signaling (189, 190).
536 Overexpression of Cx43 in HCC cells evokes downregulation of tyrosine protein kinase Src
537 and Ras-related protein Ral-A genes, which are categorized as proto-oncogenes. Both are
538 highly expressed in HCC cell lines and patient HCC tissues, and are associated with poor
539 prognosis in liver cancer due to their involvement in cell proliferation and metastasis (42, 98,
540 138, 190). The role of Cx26 is less contested and seems to be anti-tumorigenic (203).
541 Nonetheless, reports on the expression and the extent of its involvement in HCC may differ

(203). In human HCC tissue, Cx26 protein levels are unaffected, but a shift in localization towards the cytoplasm is seen (194). Others reported lowered Cx26 mRNA and/or protein expression in human HCC samples, human HCC cell lines and *in vivo* settings (93, 148, 154, 191, 202). This downregulation increases the risk of tumor recurrence in HCC patients (154). Similar to Cx32, Cx26-mediated gap junction activity is believed to play a protective role in HCC. Upon inducing the expression of Cx26 in human hepatoma HepG2 cells, recovery of gap junction activity is seen. The introduction of Cx26 contributes to morphological changes that reverse the malignant phenotype of HCC cells (203).

Conclusion

Cx channels play a prominent role in the liver. They establish a pathway for communication mechanisms. Hepatic gap junctions foresee a pathway for intercellular communication between liver cells allowing diffusion of small molecules and ions between adjacent cells. By doing so, gap junctions support liver-specific functionalities and control essential aspects of the hepatic life cycle. On the other hand, Cx hemichannels drive cellular communication between the intracellular compartment and the extracellular environment. Activation of Cx hemichannels is associated with acute and chronic liver diseases. Thus, gap junctions are seen as the “good guys’, while Cx hemichannels are considered “bad guys”. Closing of Cx hemichannels has been shown on many occasions to suppress liver toxicity and disease. Indeed, peptide-based inhibition of Cx32 and Cx43 hemichannels counteracts the clinical manifestation of acute and chronic liver disease. In this context, several pharmaceutical companies are focusing on the development of Cx hemichannel inhibitors applicable for clinical use. Moreover, Cx proteins as such play an essential role in the management of tissue homeostasis. Cx proteins control the cellular life cycle independent of their channel activities. They directly interact with mediators of cell growth and cell death control. The latter is often linked with the cytoplasmic carboxy

tail of Cx43. However, the exact role of non-channel actions in the liver needs to be clarified. Research focusing on protein-protein interactions and co-expression network analysis on Cx proteins should be encouraged, as this might unravel additional cellular processes relevant to liver homeostasis and diseases.

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Tables

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Figure legends

Figure 1: Structure of connexin (Cx) proteins and their channels. Cx proteins consist of 4 transmembrane regions, 2 extracellular loops (EL1 and EL2), 1 cytoplasmic loop (CL), an intracellular carboxy (CT) and an amino terminus (NT). Oligomerization of 6 Cx proteins creates Cx hemichannels. Homomeric hemichannels are composed of 6 single types of Cx proteins, whereas heteromeric hemichannels gather different Cx species. Gap junctions arise from the interaction of 2 Cx hemichannels on adjacent cells. Homomeric Cx hemichannels form homotypic gap junctions. Heteromeric and different Cx hemichannels generate heteromeric and heterotypic gap junctions, respectively.

Figure 2: Organization of hepatic lobules. Hepatocytes build hepatic cellular plates that diverge from the hepatic vein. Branches of the hepatic artery, hepatic portal vein and bile duct form the portal triad. Branches of the hepatic artery and portal vein gather together in hepatic capillaries that supply blood to hepatocytes. After a bidirectional exchange of substances between these hepatic capillaries and hepatocytes, blood is drained to the central hepatic vein. The localization of hepatocytes in the hepatic lobule determines access to oxygen and nutrients, whereby hepatocytes are localized in the periportal, midlobular or perivenous zone.

Figure 3: Polarization of hepatocytes. The part of the hepatocyte membrane that allows interaction with the sinusoids and the perisinusoidal space is called the ‘basal’ or ‘sinusoidal’ membrane. The part of the hepatocyte membrane facing bile canaliculi is called the ‘canalicular’ membrane. Both sides of the hepatocyte membrane are strictly separated by tight junctions, gap junctions and adherens junctions. This provides a physical barrier between the sinusoids and bile ducts that prevents mixing of blood and bile.

Figure 4: Connexin (Cx) protein expression in parenchymal and non-parenchymal cells of the liver. The most prominent Cx species is listed at the top. In hepatocytes, Cx32 is the main Cx species, while Cx43 is the most important Cx species in non-parenchymal cells.

Figure 5: Role of gap junctions in liver-specific functions. Connexin (Cx) proteins, in particular Cx32, and their channels are involved in physiological processes, such as biotransformation of xenobiotics (A), carbohydrate metabolism (B), bile production (C) and protein synthesis and secretion (D). On the one hand, Cx proteins are involved in bile production by being essential units to form a physical barrier between hepatic sinusoids and bile canaliculi, since Cx32 colocalizes with tight junctional proteins such as zonula occludens protein-1 (ZO-1). On the other hand, Cx26 and Cx32 are critical building blocks of gap junctions that allow the passage of calcium ions (Ca^{2+}) between hepatocytes, which is essential to regulate the process of bile secretion. Cx32 and its channels also stimulate the production and secretion of proteins in the liver, including albumin production. Enhanced expression of Cx26 and Cx32, associated with induced gap junction communication, but knockdown of Cx43 promotes cytochrome P450 (CYP) activity. Cx32-based gap junctions promote the propagation of hormonal signals, such as noradrenaline and glucagon, to initiate the release of glucose from the liver hepatocytes.

Figure 6: Role of gap junctions involved in the hepatic life cycle. During liver cell proliferation, connexin (Cx) protein expression levels and gap junction activity are altered (A). Increased expression of Cx26 proteins is seen until the onset of the S phase. Gap junction activity also increases in the G1 phase. The progression from the G1 to the S phase is linked with decreased levels of Cx26 and Cx32, and reduced gap junction activity. Cx43 phosphorylation occurs upon progression from the G0 phase. An apoptosis-mediating role for Cx channels is linked with their ability to spread inositol triphosphate molecules (IP_3) and

calcium ions (Ca^{2+}) to neighboring cells (B). The liver cell differentiation process of oval cells towards hepatocytes is accompanied by a switch from Cx43 to Cx32 (C).

Figure 7: Connexin (Cx) expression, Cx hemichannel activity and gap junction activity in various liver diseases. In many liver diseases, the expression of Cx26, Cx32, or Cx43 is altered. In general, while there is a decrease of Cx26 and Cx32, the opposite is observed for Cx43 in pathological situations. Cx32 and Cx43 hemichannel activity is increased in acute liver injury, non-alcoholic steatohepatitis, and fibrosis. In contrast, gap junction activity is decreased in most liver diseases.

Further reading

Gastrointestinal and liver physiology: Apoptosis and necrosis in the liver; Autophagy in the liver; Bile formation and secretion; Calcium signaling in the liver; Cellular and molecular basis of liver development; Fat soluble vitamin absorption in health and disease; Gap and tight junction physiology of the liver; Hepatic circulation (legacy); Hepatocyte polarity; Hepatocyte xenobiotic metabolism; Iron homeostasis in the liver; Overview of bile secretion (legacy); Principles of liver regeneration and growth homeostasis; Principles of membrane transport (legacy)

Cell physiology: Basic principles of transport (legacy); Cellular membranes: structural organization and basic functions.; Gap junctions; Membrane structure/proteins (legacy); Role of ion transport in control of apoptotic cell death; Roles of ion transport in control of cell proliferation